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PATENT & TRADEMARK OFFICE

EXHIBIT C
CLAIMS PENDING AFTER ENTRY OF INSTANT AMENDMENT

1. (Twice Amended) A method for determining the efficiency of an amplification of a target nucleic acid comprising the steps of:
a) preparing a dilution series of the target nucleic acid;
b) amplifying the target nucleic acid under defined reaction conditions and measuring the amplification in real-time;
c) setting a defined signal threshold value;
d) determining, for each dilution, the cycle number at which the signal threshold value is exceeded; and
e) determining the amplification efficiency as a function of the original amount of target nucleic acid.

2. (Twice Amended) The method of claim 1, further comprising determining a non-linear continuously differentiable function of a logarithm of the copy number of target nucleic acid used for the amplification as a function of the cycle number at which the signal threshold value is exceeded; and calculating the amplification efficiency from said non-linear continuously differentiable function.

3. (Twice Amended) The method of claim 1, further comprising determining a non-linear continuously differentiable function of the cycle number determined in step d) as a function of the logarithm of the copy number of target nucleic acid used for the amplification; and calculating the amplification efficiency from said non-linear continuously differentiable function.

4. (Amended) The method of claim 2, wherein the amplification efficiency E of a certain original amount of target nucleic acid is determined as the negative local first derivative of the continuously differentiable function from step (e).

5. (Amended) The method of claim 3, wherein the amplification efficiency E of a certain original amount of target nucleic acid is determined as the reciprocal negative local first derivative of the continuously differentiable function from step (e).

6. (Amended) The method of claim 2, wherein the non-linear continuously differentiable function from step (e) is determined with a polynomial fit.

7. (Amended) A method for absolute quantification of a target nucleic acid in a sample comprising the steps of:

a) determining the amplification efficiencies of the target nucleic acid and of an internal or external standard under defined amplification conditions as claimed in claim 1;

b) amplifying the target nucleic acid contained in the sample and of the internal or external standard under the same defined reaction conditions;

c) measuring the amplification of the target nucleic acid and standard in real time; and

d) calculating the original copy number in the sample by correcting the copy number derived from step c) with the amplification efficiencies determined in step a).

8. (Twice Amended) A method for quantification of a target nucleic acid in a sample relative to a reference nucleic acid comprising the steps of:

a) determining the amplification efficiencies of the target nucleic acid and of the reference nucleic acid under defined amplification conditions as claimed in claim 1;

b) amplifying the target nucleic acid contained in the sample as well as of the reference nucleic acid contained in the sample under the same defined amplification conditions;

c) measuring the amplification of the target nucleic acid and of the reference nucleic acid in real time; and

d) calculating the original ratio of target nucleic acid and reference nucleic acid in the sample by correcting the ratio derived from step c) with the amplification efficiencies determined in step a).

9. (Twice Amended) A method for quantification of a target nucleic acid relative to a reference nucleic acid and standardized with a calibrator sample comprising the steps of:

a) preparing a common or two separate dilution series of target nucleic acid and reference nucleic acid;

b) amplifying the various dilutions of target nucleic acid and reference nucleic acid under defined reaction conditions, and measuring the amplification of the nucleic acids in real-time;

c) setting defined signal threshold values for the target nucleic acid and reference nucleic acid;

d) determining the cycle numbers Cp at which the signal threshold values defined for the target nucleic acid and reference nucleic acid are exceeded in each dilution;

e) determining a continuously differentiable function of the Cp values determined in step d) as a function of the logarithm of the amounts used of target nucleic acid and determining a continuously differentiable function of the Cp values determined in step d) as a function of the logarithm of the amounts used of reference nucleic acid;

f) determining the Cp values of the target nucleic acid and reference nucleic acid in a sample to be analysed as well as in a calibrator sample;

g) assigning the Cp values measured in step f) to particular values of the functions determined in step e);

h) calculating the quotients of the function values from step g) of the target nucleic acid and reference nucleic acid for the sample to be analysed as well as for the calibrator sample; and

i) determining the ratio of the two quotients from step h) as a measure of the original amount of target nucleic acid contained in the sample to be analysed.

10. (Twice Amended) A method for quantification of a target nucleic acid relative to a reference nucleic acid and standardized with a calibrator sample comprising the steps of:

a) preparing a common or two separate dilution series of target nucleic acid and reference nucleic acid;

b) amplifying the various dilutions of target nucleic acid and reference nucleic acid under defined reaction conditions, and measuring the amplification of the nucleic acids [acid being measured] in real-time;

c) setting defined signal threshold values for the target nucleic acid and reference nucleic acid;

d) determining the cycle numbers Cp at which the signal threshold values defined for the target nucleic acid and reference nucleic acid are exceeded in each dilution;

e) determining a continuously differentiable function of the logarithm of the amounts used of target nucleic acid as a function of the Cp values determined in step d) and determining a continuously differentiable function of the logarithm of the amounts used of reference nucleic acid as a function of the Cp values determined in step d);

f) determining the Cp values of the target nucleic acid and reference nucleic acid in a sample to be analysed as well as in a calibrator sample;

g) assigning the Cp values measured in step f) to particular values of the functions determined in step e);

h) calculating the quotients of the function values from step g) of the target nucleic acid and reference nucleic acid for the sample to be analysed as well as for the calibrator sample; and

i) determining the ratio of the two quotients from step h) as a measure of the original amount of target nucleic acid contained in the sample to be analysed.

11. (Amended) The method of claim 10, wherein the continuously differentiable functions from step e) are determined with a polynomial fit.

12. (Twice Amended) The method of claim 10, wherein the amplified nucleic acids are detected with at least one fluorescently-labeled hybridization probe.

13. (Twice Amended) The method of claim 12, wherein the amplified nucleic acids are detected with FRET hybridization probes, molecular beacons, or TAQMAN® probes.

14. (Amended) The method of claim 10, wherein the amplified nucleic acids are detected with a DNA-binding dye.

15. The method of claim 6, wherein said polynomial fit is of the 3rd, 4th, 5th, 6th or 7th degree.

16. The method of claim 11, wherein said polynomial fit is of the 3rd, 4th, 5th, 6th or 7th degree.

17. (Amended) The method of claim 14, wherein said DNA-binding dye is SYBR® Green I.

18. (New) A method for determining amplification efficiency of a nucleic acid as a function of concentration comprising:
(a) amplifying different dilutions of a nucleic acid and measuring their amplification in real-time;
(b) determining the cycle number at which amplification exceeds a threshold for each of the dilutions; and
(c) determining amplification efficiency as a function of concentration of the nucleic acid.

19. (New) The method of claim 18, wherein, the amplification efficiency in step (c) is calculated from a non-linear, continuously differentiable function of the cycle number determined in step (b) that maps said cycle number to the logarithm of concentration of the target nucleic acid.

20. (New) The method of claim 18, wherein, the amplification efficiency in step (c) is calculated from a non-linear, continuously differentiable function of the logarithm of concentration of nucleic acid that maps said logarithm of concentration to the cycle number determined in step (b).

21. (New) The method of claim 19, wherein the amplification efficiency is determined as the negative local first derivative of the continuously differentiable function.

22. (New) The method of claim 20, wherein the amplification efficiency is determined as the reciprocal negative local first derivative of the continuously differentiable function.

23. (New) The method of claim 19, wherein the non-linear, continuously differentiable function is determined with a polynomial fit.

24. (New) The method of claim 23, wherein said polynomial fit is of the 3rd, 4th, 5th, 6th or 7th degree.

25. (New) The method of claim 20, wherein the non-linear, continuously differentiable function is determined with a polynomial fit.

26. (New) The method of claim 26, wherein said polynomial fit is of the 3rd, 4th, 5th, 6th or 7th degree.

27. (New) A method for calculating the concentration of a target nucleic acid in a sample to be analysed, wherein the sample to be analysed comprises the target nucleic acid and a reference nucleic acid in a single vessel, comprising:

(a) amplifying different dilutions of the target nucleic acid and different dilutions of the reference nucleic acid and determining amplification efficiencies of the target nucleic acid and the reference nucleic acid, wherein the amplification efficiency of the target nucleic acid and the reference nucleic acid are each independently determined according to the method of claim 18;

(b) amplifying the target nucleic acid and the reference nucleic acid and measuring amplification in real-time; and

(c) calculating the concentration of target nucleic acid by correcting the concentration of target nucleic acid obtained in step (b) with concentration of reference nucleic acid

obtained in step (b) and the amplification efficiencies of target nucleic acid and reference nucleic acid determined in step (a).

28. (New) A method for calculating the concentration of a target nucleic acid in a sample to be analysed, wherein the sample to be analysed comprises the target nucleic acid and a reference nucleic acid in separate vessels, comprising:
(a) amplifying different dilutions of the target nucleic acid and different dilutions of the reference nucleic acid and determining amplification efficiencies of the target nucleic acid and the reference nucleic acid, wherein the amplification efficiency of the target nucleic acid and the reference nucleic acid are each independently determined according to the method of claim 18;
(b) amplifying the target nucleic acid in the sample to be analysed and measuring amplification in real-time;
(c) amplifying the reference nucleic acid in the sample to be analysed under amplification conditions similar to those in step (b) and measuring amplification in real-time; and
(d) calculating the concentration of target nucleic acid by correcting the concentration of target nucleic acid obtained in step (b) with concentration of reference nucleic acid obtained in step (c) and the amplification efficiencies of target nucleic acid and reference nucleic acid determined in step (a).

29. (New) A method for calculating the ratio of concentration of a target nucleic acid relative to a reference nucleic acid in a sample to be analysed, wherein the sample to be analysed comprises the target nucleic acid and the reference nucleic acid in a single vessel, comprising:
(a) amplifying different dilutions of the target nucleic acid and different dilutions of the reference nucleic acid and determining amplification efficiencies of the target nucleic acid and the reference nucleic acid, wherein the amplification efficiency of the target nucleic acid and the reference nucleic acid are each independently determined according to the method of claim 18;

(b) amplifying the target nucleic acid and the reference nucleic acid in the sample to be analysed and measuring amplification in real-time;

(c) calculating an initial ratio of concentration of the target nucleic acid relative to the reference nucleic acid from the concentrations of the target nucleic acid and the reference nucleic acid obtained in step (b); and

(d) calculating the ratio of concentration of the target nucleic acid relative to the reference nucleic acid by correcting the initial ratio obtained in step (c) with the amplification efficiencies for target nucleic acid and reference nucleic acid determined in step (a).

30. (New) A method for calculating the ratio of concentration of a target nucleic acid relative to a reference nucleic acid in a sample to be analysed, wherein the sample to be analysed comprises the target nucleic acid and the reference nucleic acid in separate vessels, comprising:

(a) amplifying different dilutions of the target nucleic acid and different dilutions of the reference nucleic acid and determining amplification efficiencies of the target nucleic acid and the reference nucleic acid, wherein the amplification efficiency of the target nucleic acid and the reference nucleic acid are each independently determined according to the method of claim 18;

(b) amplifying the target nucleic acid in the sample to be analysed and measuring amplification in real-time;

(c) amplifying the reference nucleic acid in the sample to be analysed under amplification conditions similar to those in step (b) and measuring amplification in real-time; and

(d) calculating an initial ratio of concentration of the target nucleic acid relative to the reference nucleic acid from the concentration of the target nucleic acid obtained in step (b) and the concentration of the reference nucleic acid obtained in step (c); and

(e) calculating the ratio of concentration of the target nucleic acid relative to the reference nucleic acid by correcting the initial ratio obtained in step (d) with the amplification efficiencies for target nucleic acid and reference nucleic acid determined in step (a).

31. (New) A method for quantifying a target nucleic acid relative to a reference nucleic acid in a sample to be analysed, wherein the sample to be analysed comprises the target nucleic acid and the reference nucleic acid, comprising:

(a) amplifying different dilutions of the target nucleic acid and different dilutions of the reference nucleic acid and measuring amplification in real-time;

(b) determining the cycle number at which amplification exceeds a first threshold for each of the dilutions of step (a);

(c) generating a continuously differentiable target function, F_T, of target nucleic acid cycle number and a continuously differentiable reference function, F_R, of reference nucleic acid cycle number wherein:

F_T maps the cycle numbers determined in step (b) for the dilutions of the target nucleic acid to the logarithm of concentration of the target nucleic acid and

F_R maps the cycle numbers determined in step (b) for the dilutions of the reference nucleic acid to the logarithm of concentration of the reference nucleic acid;

(d) amplifying the target nucleic acid and the reference nucleic acid in the sample to be analysed under similar amplification conditions and measuring amplification in real-time;

(e) amplifying a calibrator sample and measuring amplification in real-time, wherein the calibrator sample comprises the target and reference nucleic acids in a known concentration ratio;

(f) determining F_T(Cp-Tar), F_R(Cp-Ref), F_T(Cp-Tar_{cal}) and F_R(Cp-Ref_{cal}), wherein:

F_T(Cp-Tar) is the value of the target function, F_T, at the cycle number (Cp-Tar) at which the amplification exceeds a second threshold for the target nucleic acid in step (d),

F_R(Cp-Ref) is the value of the reference function, F_R, at the cycle number (Cp-Ref) at which the amplification exceeds the second threshold for the reference nucleic acid in step (d),

F_T(Cp-Tar_{cal}) is the value of the target function, F_T, at the cycle number (Cp-Tar_{cal}) at which the amplification exceeds the second threshold for the target nucleic acid in step (e) and

$F_R(Cp\text{-}Ref_{cal})$ is the value of the reference function, F_R, at the cycle number ($Cp\text{-}Ref_{cal}$) at which the amplification exceeds the second threshold for the reference nucleic acid in step (e),

and wherein the second threshold can, but need not be, identical to the first threshold;

(g) quantifying the amount of target nucleic acid relative to the reference nucleic acid, wherein, the relative amount is:

$$\frac{F_T(Cp\text{-}Tar) / F_R(Cp\text{-}Ref)}{F_T(Cp\text{-}Tar_{cal}) / F_R(Cp\text{-}Ref_{cal})}$$

32. (New) The method of claim 31, wherein the continuously differentiable function is determined with a polynomial fit.

33. (New) The method of claim 32, wherein said polynomial fit is of the 3rd, 4th, 5th, 6th or 7th degree.

34. (New) The method of claim 31, wherein the amplified nucleic acid is detected with at least one fluorescently labeled hybridization probe.

35. (New) The method of claim 34, wherein the amplified nucleic acid is detected with FRET hybridization probes, molecular beacons, or TAQMAN® probes.

36. (New) The method of claim 31, wherein the amplified nucleic acid is detected with a DNA-binding dye.

37. (New) The method of claim 36, wherein said DNA-binding dye is SYBR® Green I.